JOURNAL OF BACTERIOLOGY, Aug. 2007, p. 5825–5838 Vol. 189, No. 16
0021-9193/07/$08.00+0 doi:10.1128/JB.00242-07

Two-Component Response Regulators of *Vibrio fischeri*: Identification, Mutagenesis, and Characterization▽

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Received 13 February 2007/Accepted 8 June 2007

Two-component signal transduction systems are utilized by prokaryotic and eukaryotic cells to sense and respond to environmental stimuli, both to maintain homeostasis and to rapidly adapt to changing conditions. Studies have begun to emerge that utilize a large-scale mutagenesis approach to analyzing these systems in prokaryotic organisms. Due to the recent availability of its genome sequence, such a global approach is now possible for the marine bioluminescent bacterium *Vibrio fischeri*, which exists either in a free-living state or as a mutualistic symbiont within a host organism such as the Hawaiian squid species *Euprymna scolopes*. In this work, we identified 40 putative two-component response regulators encoded within the *V. fischeri* genome. Based on the type of effector domain present, we classified six as NarL type, 13 as OmpR type, and six as NtrC type; the remaining 15 lacked a predicted DNA-binding domain. We subsequently mutated 35 of these genes via a vector integration approach and analyzed the resulting mutants for roles in bioluminescence, motility, and competitive colonization of squid. Through these assays, we identified three novel regulators of *V. fischeri* luminescence and seven regulators that altered motility. Furthermore, we found 11 regulators with a previously undescribed effect on competitive colonization of the host squid. Interestingly, five of the newly characterized regulators each affected two or more of the phenotypes examined, strongly suggesting interconnectivity among systems. This work represents the first large-scale mutagenesis of a class of genes in *V. fischeri* using a genomic approach and emphasizes the importance of two-component signal transduction in bacterium-host interactions.

The symbiosis between the Hawaiian squid *Euprymna scolopes* and the bacterium *Vibrio fischeri* serves as a model for symbiotic bacterium-host interactions. Previous studies have revealed a number of bacterial factors required for host colonization (24, 56), including motility (25, 49, 57) and luminescence (78). Many of these factors were identified by generating and testing specific hypotheses developed from an understanding of the general colonization process. To date, however, large-scale studies of colonization factors have been hampered by a deficit of genetic tools needed for bacterial mutant construction. Recently, however, the genome sequence of *V. fischeri* has been published (65). In addition, the availability of a useful suicide vector (17) has greatly facilitated mutant construction (73, 90). Thus, it is now possible to approach the investigation of *V. fischeri* biology from a genomic perspective.

One large-scale approach to understanding the biology of an organism is to mutagenize regulatory genes (such as two-component response regulators [RRs]), as each mutation potentially impacts the expression and/or function of a number of proteins. Both gram-negative and gram-positive bacteria utilize two-component signal transduction systems to sense and respond to different environmental stimuli, such as nutrient availability, pH, osmolarity, and host factors (5, 10). These signaling pathways are known to influence numerous processes in bacteria, including motility, bioluminescence, biofilm formation, and pathogenesis (31). Simple two-component signal transduction systems utilize two main protein components: (i) the sensor histidine kinase protein (SK), which functions as a dimer to autophosphorylate at a conserved histidine residue (using ATP as a phosphodonor) in response to a specific signaling event, and (ii) the RR, which catalyzes transfer of phosphate from the cognate SK to a conserved aspartate residue on its receiver or REC domain. More-complex "hybrid" systems consist of multiple phosphoryl-accepting histidine and aspartate residues within the same pathway. Regardless of the complexity of the upstream signal transduction pathway, the RR ultimately changes its activity upon phosphorylation to elicit a cellular response to the initial signal.

Typically, RRs contain two functional domains, a REC domain that participates in signal transduction and an effector domain necessary for the cellular response. Residues in the activation domain that are highly conserved among RRs provide a basis for identifying putative RRs. Studies of a number of such regulators, such as the well-characterized CheY, provide a basis for understanding the function of conserved residues in the activation domain. In *Escherichia coli* CheY, six amino acid residues play key roles in activation. Phosphorylation occurs on Asp57 (9), while two additional Asp residues, at positions 12 and 13, chelate a Mg^{2+} ion necessary for transfer of the activating phosphoryl group from the donor His (81). Three other amino acids, Thr87, Tyr106, and Lys109, convey the phosphorylation-associated conformational change that facilitates effector function (6, 12, 37, 38, 70).

In addition to their conserved activation domains, RRs contain effector domains responsible for eliciting a cellular re-

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▽ Published ahead of print on 22 June 2007.

sponse to stimulation. Non-DNA-binding RRs (such as CheY) may directly impact protein function by affecting, for example, folding or stability; as a result they may also indirectly impact transcription of downstream target genes. DNA-binding RRs typically fall into three distinct subclasses, exemplified by NarL, OmpR, and NtrC (74). Members of each subclass maintain unique DNA sequence specificities and mechanisms of action. Members of the NarL subclass contain a classical helix-turn-helix (HTH) DNA-binding motif (4). Those of the OmpR subclass share a winged helix-turn-helix (wHTH) motif. In this latter motif, the traditional recognition helix used in major groove interactions with DNA is flanked by extended looped regions, which may interact with the minor groove of the target DNA sequence (46). The third, NtrC-like subclass consists of a structurally distinct group of RR proteins that serve as coactivators for RNA polymerase holoenzymes associated with the alternative sigma factor σ^{54}, and members of this subclass are known as σ^{54}-dependent RRs. These proteins contain two distinct effector domains. One domain binds DNA, while the other, approximately 240 amino acid residues in length, is responsible for oligomerization and ATP hydrolysis. Together with RNA polymerase containing σ^{54}, the two RR domains promote open complex formation during transcriptional initiation (54, 55, 59, 68, 80).

The importance of two-component regulators is underscored by their abundance in many sequenced bacterial genomes. For example, *E. coli* contains 32 two-component regulators (53). In *V. fischeri*, only five RRs have been described thus far: GacA, LuxO, ArcA, SypG, and SypE (8, 52, 83, 84, 90); of these, only the first three have been characterized by genetic disruption. Mutants that lack GacA exhibit pleiotropic effects in culture, fail to initiate symbiotic colonization (84), and fail to induce host development (83). LuxO and ArcA both serve as negative regulators of luminescence genes (8, 40–42, 52); however, of these two, only LuxO appears necessary during symbiotic initiation, suggesting that it controls symbiosis genes in addition to those governing luminescence (40–42, 52). Given that so few of these regulators have been characterized, we chose to identify, mutagenize, and characterize specific phenotypes mediated by *V. fischeri* RRs. This work demonstrates the feasibility of performing global screens for gene function in *V. fischeri* and represents an initial characterization of many two-component signal transduction pathways in this organism.

MATERIALS AND METHODS

Bioinformatics and statistics. Amino acid sequences of the *E. coli* K12 RRs CheY (47), OmpR (86), NarL (26), and NtrC (51) were obtained from the NCBI database and utilized as query sequences to search the genome of *V. fischeri* strain ES114 in the ERGO light database from Integrated Genomics (http://www.ergo-light.com/ERGO/). *V. fischeri* open reading frames (ORFs) that exhibited an expected threshold, or "E," value of <1 were further subjected to rpsBLAST analysis (44) to identify conserved domains. ORFs containing REC signal receiver domains were compared to CheY and other *E. coli* RR sequences by ClustalW amino acid alignment (76) to screen for conserved residues within this domain (D12, D13, D57, T87, Y106, and K109) (81). Putative RRs were then classified by using rpsBLAST (44) and ClustalW (76) analysis of each RR effector domain. Where indicated, Student's *t* tests (unpaired) were performed to generate a probability, or *P*, value to determine significance.

Strains and media. *V. fischeri* strain ES114 was the parent strain used in this study (7). *V. fischeri* strain KV1421 (58), which contains a chromosomal erythromycin resistance cassette (Tn*7*::*erm*) (48), was used as a control for motility and bioluminescence experiments. *E. coli* strains DH5α*λpir* (17), CC118*λpir* (29), and TAM1*λpir* (Active Motif, Carlsbad, CA) were used for cloning. Triparental matings to introduce plasmid DNA into *V. fischeri* utilized *E. coli* strain CC118*λpir* carrying the conjugation helper plasmid pEVS104 (73).

V. fischeri strains were grown in the following media. To test growth in a minimal medium, HEPES-MM was used (64). For routine culturing, either LBS (72) or SWT medium was used; the latter contains 0.5% tryptone, 0.3% yeast extract, and seawater salts (90). For motility experiments, cells were grown in TB-SW, a tryptone-based medium containing seawater salts, to maintain consistency with previously reported assays of *V. fischeri* motility (15). Previous work within the field indicates that increased medium osmolarity correlates with increased luminescence in culture (71). Thus, additional NaCl was added to SWT to a final concentration of 510 mM to assay for bioluminescence; we designated this medium as SWTS. *E. coli* strains were grown in LB (14), brain heart infusion (Difco), or SOC (66) medium. Where appropriate, antibiotics were added to the following final concentrations: chloramphenicol at 25 μg ml^{-1} for *E. coli* and 2.5 μg ml^{-1} for *V. fischeri*, kanamycin at 50 μg ml^{-1}, and erythromycin (ERY) at 5 μg ml^{-1} for *V. fischeri* and 150 μg ml^{-1} for *E. coli*. Agar was added to a final concentration of 1.5% for solid media.

Molecular and genetic techniques. Standard molecular biology techniques were used for all plasmid constructions. Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). To generate vector integration mutations (11) in each RR, DNA oligonucleotides used for amplifying internal fragments of RR genes (Table 1) were obtained from MWG Biotech (High Point, NC). Amplified PCR fragments were cloned into the *oriR6K*-based suicide vector pEVS122 (17), which was digested with the restriction enzyme SmaI. In some cases, internal fragments of RRs first were cloned using the TOPO TA cloning kit per the manufacturer's instructions (Invitrogen, Carlsbad, CA) and then subsequently subcloned into either pEVS122 or its derivative, pESY20 (58). Strains of *E. coli* carrying internal fragments of each RR in pEVS122 or pESY20 were used in triparental conjugations to introduce the DNA into wild-type *V. fischeri* strain ES114. ERY-resistant colonies that arose were presumptive RR mutants. To confirm each mutant, Southern blot analysis was performed as previously described (79) using digoxigenin-labeled pEVS122 as a probe to detect complementary sequences (Roche Molecular Biochemicals, Indianapolis, IN). Hybridization and detection were carried out as described previously (79). RR mutants resulting from these manipulations are shown in Table 2.

Stability of the duplication. Vector integration mutagenesis results in a partial duplication of the interrupted gene, and thus, recombination to yield a wild-type strain can occur. To determine whether such an event is frequent, we grew three *V. fischeri* RR mutants (KV1548, KV1703, and KV1704) for at least 10 generations in SWT in the absence of ERY selection. Cells were diluted and inoculated onto SWT plates (also nonselective). More than 200 colonies from each of the strains in triplicate were then patched onto LBS containing ERY and SWT plates to determine the percentage that retained ERY resistance.

Luminescence assays. To assay for potential bioluminescence defects, the RR mutants and ES114 were grown overnight in SWT- and subcultured into SWTS to a starting optical density at 600 nm (OD_{600}) of approximately of 0.03. At various times after inoculation, 1-ml samples were taken for luminescence and OD measurements. A TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) set at factory settings was used to determine the relative light units integrated over a 6-second count.

Motility assays. To assay motility, KV1421 (58) and individual RR mutants were grown to the mid-exponential phase of growth (OD, ~0.3) in TB-SW, and 10-μl aliquots were inoculated onto TB-SW plates containing 0.225% agar (15, 85). Each mutant was spotted onto an individual plate along with a wild-type control. Over the course of 4 to 5 hours of incubation at 28°C, the diameter of migrating rings was measured.

Competitive symbiosis and growth assays. To assess the colonization capability of mutant *V. fischeri* strains, juvenile squid were inoculated into artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) containing between 1,000 and 6,000 cells per ml of seawater. For competitive colonization assays, animals were inoculated with an approximately 1:1 ratio of mutant and wild-type cells. In the individual inoculation experiment, animals were inoculated with ES114 or KV1787 (Δ*sypG*) alone. The presence of bacterial strains within the light organs of juvenile squid was assessed through luminescence and homogenization/plating assays as described previously (63). The limit of detection based on these methods is 14 *V. fischeri* cells per squid. In competitive assays, colonization is reported as the log-transformed relative competitive index (log RCI). This number is generated by dividing the ratio of mutant to wild-type cells within the homogenate by the ratio present in the inoculum and subsequently determining the $\log_{10}$ value of that number.

TABLE 1. Primers used in this study

ORF name	Primer sequence	Primer name
VF0095	GACCTTAACTAGCACCAATATG	3153-c-F
	GATGAGCGAGATAAGCGTCC	3153-c-R
VF0114	CGTTACTTGTCAGAACAAGGC	155-F
	GAACATTTCACGTGTGCCTAG	155-R
VF0454	AAAAAGGTACCCATTGCAACGTTAGTTAATAAC	2061intF[a]
	TTTTTGGATCCTCTAAATCGATGTGGCTTGC	2061intR[a]
VF0526	AGGGACACGTTGTTTATTCTG	2425-c-F
	GTGTAAATCCGGCTGCTCG	2425-c-R
VF0923	CTACTGGGATGATTGCCGC	4095 F
	GCGAATGTATGAATAAGGGGAG	4095 R
VF0937	AAAAAGGTACCCGGCTCATGGCTCAATTG	LuxOintF[a]
	TTTTTGGATCCTCTAGCCAAGGGTCTCGG	LuxOintR[a]
VF1054	TCTGATTTGTGTGGACGACC	1666-c-F
	GATAGTCATGAGATACATCCG	1666-c-R
VF1148	ATGAACTCTTTGCTAGAGAAG	2414-c-F
	TATCTGGTTGGCTAAGCGG	2414-c-R
VF1396	CGCACAAATTGTAGAAACCC	459-c-F
	GGAACTGCGGGTATCTAAG	459-c-R
VF1401	GAAATTGTATGCAAACCATAC	1925-c-F
	ATTAGATGGAGCAAGTTGAGC	1925-c-R
VF1570	ACGAAGTGGTAACTCGCTC	1334-c-F
	CGAACAACGTTAGACTCATC	1334-c-R
VF1689	AAAAAGGTACCGTAGAGGAAGCGGTATTGG	2073intF[a]
	TTTTTGGATCCTATGGATGCCACCATTGCC	2073intR[a]
VF1783	AAAAAGGTACCCCTCGTTTAGAGGCGATTG	796intF[a]
	TTTTTGGATCCTGGTTGTCATCACCAATACG	796intR[a]
VF1854	CGTGATGGTGCTTGGTCC	5201F
	TCATCCGCAGTGATCGTATG	5201R
VF1909	CATCCGTTAATGCGTCGTGG	3374-c-F
	TTGCCGCCTTTCATCGCTTC	3374-c-R
VF1988	GAAGCACCAATTCGTGAAATG	846-c-F
	GTCACACGATGAGATACAGG	846-c-R
VF2120	GAACACGTAACTCGTAATACG	998-c-F
	AACCGTTGAACACATAGCGC	998-c-R
	GTTACGTGCACGAATAGTCAG	998-c-R2
VF2343	GATATTGAACTGACAGCTCTG	4741-c-F
	TGATACTACTGCTATTACTCTC	4741-c-R
VF2374	CTGCTCTTAGACTTAAAAATGCC	65F
	CTTTATATTGCTGAGGCGTTAG	65R
VFA0041	ACCACATCATTGTCCGCTC	1605-c-F
	CGCAATATCTGGCGTAAGG	1605-c-R
VFA0103	GGTTGATGATGAGCTCTCAG	3280-c-F
	CTCTTCACCATCCACTGGC	3280-c-R
VFA0179	AAAGGGATTAACAGAGTCAGG	3211-c-F
	GGTTTCTTGTGGCGGTTCG	3211-c-R
VFA0181	GAAGTATTACAAGATGAAGGC	3208-c-F
	CCTACATAGCTTTGACGCTG	3208-c-R
VFA0211	AAAAAGGTACCGGATCTAAAGGATTATCAATGGCG	3110intF[a]
	TTTTTGGATCCAAGACAAATGCGATTCAATGG	3110intR[a]
VFA0216	AAAGTAATGGACCTTGCTGAAGTC	3116F
	GATATGCGAACTCCATCTGAGAG	3116R
VFA0266	ACCATGTAGCAGAAACCATTG	629-c-F
	GGCGATATAACGTGCCTGC	629-c-R
VFA0436	CATGCTACCTAATCTTGATGGG	1948F
	ACCACTTCATCAGCATTTTCC	1948R
VFA0561	CGGACGGACTGATTGTCAG	140-c-F
	ACGTTCTCACCTGAATTAACC	140-c-R
VFA0608	TCCGCTCTGGCTCGTAAAC	737-c-F
	GCTACGTTAGCTACCTCAAG	737-c-R
VFA0732	ATGAGCCATTATTACGCTTTC	3084-c-F
	GCTGTGCAGACAGTTGCTG	3084-c-R
VFA0795	CGCTCGGAAATCATTAGCTC	2872-c-F
	GAACGATGCAATGTAGCGAC	2872-c-R
VFA0862	AAAAAGGTACCGGACGTTGTTTTCCACCAC	3758intF[a]
	TTTTTGGATCCACGGACATTCACCATCACG	3758intR[a]
VFA1012	AAAAAGGTACCCCTATCATGGGAGGGTTTG	3034intF[a]
	TTTTTGGATCCGCTGTTTGCCTTAATTGCTC	3034intR[a]
VFA1017	CTCAGATAGATAAAGCGTATTC	3040-c-F
	AATGCTTGAACGAATAGTGGG	3040-c-R
VFA1024	CAATGGCAGAGAAGCGCTG	3630-c-F
	TGTGTGAGGTCTGCATGTTG	3630-c-R
VFA1026	CCACTTCTCTGGCTATCCTC	3633-c-F
	CTCGGCGCATACTTCTTTAC	3633-c-R

[a] These primers contain additional sequences with engineered restriction sites.

TABLE 2. *V. fischeri* strains used in this study

Strain	Genotype	Reference
ES114	Wild type	7
KV1421	*att*Tn*7*::Ery^r	58
KV1548	VF2120 (*arcA*)::pEAH1	This study
KV1585	VF1570 (*torR*^{a})::pKV174	This study
KV1592	VFA1024 (*sypE*)::pEAH7	This study
KV1593	VFA0179::pKV178	This study
KV1594	VF1401::pKV177	This study
KV1595	VF1396 (*phoP*^{a})::pKV176	This study
KV1596	VFA0561::pKV175	This study
KV1612	VFA1017::pKV179	This study
KV1640	VFA0041 (*uhpA*^{a})::pTMB26	This study
KV1641	VF1054::pAIA1	This study
KV1650	VFA0266::pTMB27	This study
KV1651	VF1988 (*phoB*^{a})::pTMB28	This study
KV1654	VFA1012::pTMB31	This study
KV1655	VF2343 (*cpxR*^{a})::pTMB32	This study
KV1665	VF1909 (*narP*^{a})::pTMB33	This study
KV1666	VFA1026 (*sypG*)::pAIA4	This study
KV1668	VFA0211::pAIA6	This study
KV1672	VFA0181::pTMB34	This study
KV1703	VF2120 (*arcA*)::pTMB35	This study
KV1704	VF2120 (*arcA*)::pTMB36	This study
KV1714	VFA0795::pEAH10	This study
KV1715	VF0454 (*vpsR*^{a})::pEAH11	This study
KV1727	VF0526 (*phoP*^{a})::pEAH4	This study
KV1730	VF0095 (*ntrC*^{a})::pKV180	This study
KV1787	Δ*sypG*	This study
KV1809	VF1854 (*flrC*^{a})::pEAH26	This study
KV2164	VF2374::pEAH24	This study
KV2165	VFA0216::pEAH25	This study
KV2191	VF0937 (*luxO*^{a})::pAIA3	This study
KV2501	VF1689 (*expM*^{a})::pAIA2	This study
KV2503	VFA0103::pAIA5	This study
KV2505	VFA0802 (*cheV*^{a})::pKV207	This study
KV2507	VF0114 (*ompR*^{a})::pKV209	This study
KV2509	VFA0698 (*cheV*^{a})::pKV214	This study
KV2510	VF1833 (*cheY*^{a})::pKV215	This study
KV2636	VF1148 (*yehT*^{a})::pTMB30	This study
KV2637	VF1879 (*cheV*^{a})::pKV216	This study
KV2874	VFA0732::pKV208	This study

[a] Putative identity based on an e-value less than e^{-20}, per BLAST analysis, compared with regulators previously characterized in *E. coli*, *B. subtilis*, or *V. cholerae*.

To assess competitive growth in culture, *V. fischeri* RR mutant and marked wild-type strains were coinoculated into SWT medium at an approximately 1:1 ratio and the inoculum was plated. After at least 20 generations of growth in SWT, cells were diluted and plated again. The log RCI was calculated as described above.

RESULTS

Identification of putative RR proteins in the *V. fischeri* genome. To identify *V. fischeri* ORFs encoding putative RRs, we searched the genome for characteristic signal receiver (REC) domains and conserved residues therein (further detailed in Materials and Methods). In CheY, these conserved residues have been characterized as follows: the site of phosphorylation (Asp57), two additional aspartate residues (Asp12 and Asp13) necessary for maintaining a Mg^{2+} ion within the active site (81), and three residues (Thr87, Tyr106, and Lys109) that facilitate effector function (3, 12, 39). By these analyses, we identified 40 putative RR genes. For simplicity, we will refer to these proteins as RRs rather than putative RRs, although final confirmation that these identified proteins in fact function as RRs will require biochemical characterization not part of the current study. We further categorized the RR proteins into four main groups based on the nature of their predicted effector domains, represented by the following proteins: (i) CheY, which lacks a DNA-binding domain; (ii) NarL, which includes an HTH DNA-binding domain; (iii) OmpR, which contains a wHTH DNA-binding domain; and (iv) NtrC, with both a nucleotide binding domain and an HTH DNA-binding domain (Tables 3 to 6).

The first group of RRs, shown in Table 3, includes 15 ORFs that encode typical REC domains and, like CheY, lack distinct DNA-binding motifs. The *V. fischeri* homolog of CheY is likely encoded by VF1833. This gene is linked to other putative chemotaxis genes, including the RR genes *cheB* (VF1830) and *cheV* (VF1879). There exist two other CheV-like proteins (encoded by VFA0698 and VFA0802), which display 33.9% and 48.4% identity, respectively, to the VF1879-encoded CheV. These genes are present at distinct locations on chromosome 2, unlinked to any other genes involved in flagellar biosynthesis or chemotaxis (most of which are encoded on chromosome 1). Another RR in this group is *sypE* (VFA1024), a member of the recently identified *syp* cluster of genes involved in cell surface phenotypes and colonization (89, 90). SypE itself has yet to be characterized. The additional proteins in this group do not exhibit significant sequence similarity to known regulators.

The RRs in the second group contain, in addition to REC domains, HTH DNA-binding motifs found in RRs such as NarL (26) (Table 4). This class of six proteins includes GacA, as well as proteins with sequence similarity to known *E. coli* RRs. These include potential homologs of NarP, a regulator of genes involved in anaerobic respiration during nitrate limitation (61); UhpA, which regulates hexose phosphate transport (23, 32, 82); and YehT, an RR involved in multidrug resistance (30).

The third group contains 13 RR proteins that have a predicted wHTH DNA-binding domain, a motif found in RRs such as the outer membrane porin regulator OmpR (45, 80) (Table 5). Based on sequence similarity, VF0114 is likely an OmpR homolog. The only member of this class that has been characterized in *V. fischeri* is ArcA (8). Other RRs in this group exhibit similarity to characterized proteins, such as TorR, a regulator of the TMAO anaerobic respiratory system (69); PhoB, a phosphate regulator (43); and CpxR, an envelope stress regulator (16). The additional eight RRs within this family have no predicted homologs that have been characterized.

The fourth group consists of six putative σ^{54}-dependent RRs with sequence similarity to the nitrogen regulator NtrC (Table 6). In addition to REC and DNA-binding domains, proteins in this class contain a nucleotide binding domain required for σ^{54}-dependent transcriptional activation (59, 80). In addition to an NtrC homolog, likely encoded by VF0095, this group includes the luminescence regulator LuxO and VF1854, a putative homolog of the flagellar synthesis regulator FlrC, based on similarity to the *Vibrio cholerae* protein (34). SypG is another σ^{54}-dependent activator, which regulates expression of the *syp* cluster of genes (90). VF0454 exhibits similarity to the *V. cholerae* regulator of polysaccharide synthesis, VpsR. VF1401 has no previously characterized putative homolog.

TABLE 3. Putative RRs with no clear DNA-binding motif

ORF[a]	Length (amino acids)	Identity to CheY (%)[b]	Conserved residue[c]						Known or possible identity[d]	% Identity/ similarity[e]	Possible SK partner[f]
			D12	D13	D57	T87	Y106	K109			
VF0923	439	20.9	X	X	X			X			Unpaired
VF1054	157	26.4	X	X	X		X	X			VF1053
VF1689	368	23.3	X	X	X			X	ExpM[g]	32/53	Unpaired
VF1830	376	23.3	X	X	X				CheB	35/54	VF1831 (CheA)
VF1833	122	65.1	X	X	X	X	X	X	CheY	68/86	VF1831 (CheA)
VF1879	306	32.6	X	X	X	X		X	CheV[h]	30/55	VF1831 (CheA)
VFA0216	382	24.8	X	X	X						Unpaired
VFA0608	334	24.0	X	X	X			X			Unpaired
VFA0698	298	25.6	X	X	X	X		X	CheV[h]	28/53	Unpaired
VFA0732	267	22.5	X	X	X		X	X			VFA0733
VFA0795	331	23.3	X	X	X			X			Unpaired
VFA0802	313	27.1	X	X	X	X		X	CheV[h]	31/53	Unpaired
VFA1012	299	24.8		X	X	X		X			Unpaired
VFA1017	572	23.3	X	X	X		X	X			VFA1016
VFA1024	505	21.7	X	X	X			X	SypE[i]		VFA1025 (SypF)

[a] ORFs are designated VF if located on chromosome 1 and VFA if found on chromosome 2.
[b] Percent identity to *E. coli* CheY protein calculated by determining the number of identical residues per ClustalW alignment (76) and dividing by the number of amino acids in the shorter sequence.
[c] Presence (X) of amino acid residues conserved within REC domains of RR proteins (numbers shown represent placement within *E. coli* CheY).
[d] Possible identities were assigned to proteins whose e-values to the indicated *E. coli* (or alternative species, where indicated) gene were more significant than e^{-20}, per BLAST analysis (1, 67).
[e] Per BLAST alignment (bl2seq) using the Blosum 62 matrix across the length of the *V. fischeri* protein (28, 75).
[f] Identified as SK based on the presence of conserved histidine (H box) motifs by rpsBLAST analysis (44); putative homology, based on parameters outlined in footnote *d*, is indicated in parentheses.
[g] Compared to *Erwinia carotovora* sequence (2).
[h] Compared to *Bacillus subtilis* sequence (22).
[i] SypE has been previously named (90).

The genes of cognate RR and SK pairs are often physically linked on the chromosome. Therefore, we extended our analysis of RR genes to examine the functions of nearby genes. Using BLAST analysis, genes encoding the conserved SK domain involved in dimerization and phosphorylation, termed HisKA, were considered putative SKs. Interestingly, each of the RRs within the OmpR-like family is linked to a putative SK (Table 5). The RRs of the HTH and σ^{54}-dependent families each contain five (of six) seemingly paired members (Tables 4 and 6). In contrast, only 7 of the 15 RRs listed in Table 3 (lacking a DNA-binding motif) appear linked to SK genes.

Finally, we analyzed the distribution of RRs across each of *V. fischeri*'s two chromosomes. The larger chromosome, chromosome 1 (2.9 Mb, designated VF), encodes 22, or about half, of the RRs, 16 of which have known or tentatively assigned identities. The remaining 18 RRs are encoded on the smaller chromosome, chromosome 2 (1.3 Mb, designated VFA), and only five of them have assigned identities. Thus, in addition to the imbalance in the number of RRs with tentative assignments, the proportion of RRs on each chromosome is not representative of the size of the respective chromosomes. With two exceptions, we have not confirmed the putative identities of these genes; however, to aid the reader, we will include the putative assignments of the RR genes in parentheses following their gene numbers throughout this work.

Construction of RR mutants. To begin to characterize the two-component signal transduction systems of *V. fischeri*, we constructed mutant strains defective for individual *V. fischeri* RR genes. We chose to eliminate from our study the RR *gacA* gene (VF1627), as it has been characterized extensively, but included *luxO* (VF0937) and *arcA* (VF2120) as controls.

To mutate the RR genes with relative ease, we cloned an internal portion of each RR gene into a suicide vector (pEVS122 [17] or pESY20 [58]), marked with ERY resistance (Ery^r). Recombination of these constructs into the *V. fischeri* genome generates two truncated copies of the gene, which generally results in a null mutation (11). Although such mutations have the potential for polar effects on downstream genes

TABLE 4. Putative HTH RRs[a]

ORF	Length (amino acids)	Identity to CheY (%)	Conserved residue						Known or possible identity	% Identity/ similarity	Possible SK partner
			D12	D13	D57	T87	Y106	K109			
VF1148	238	28.7	X	X	X			X	YehT	51/73	VF1149 (YehU)
VF1627	214	28.7	X	X	X		X	X	GacA[b]		VF2082 (GacS)
VF1909	203	29.5	X	X	X	X	X	X	NarP	54/72	VF1908 (NarQ)
VF2374	209	23.3	X	X	X			X			Unpaired
VFA0041	202	24.8	X	X	X			X	UhpA	64/75	VF0040 (UhpB)
VFA0103	205	24.8	X	X	X	X		X			VFA0102

[a] See Table 3 footnotes for explanations of column heads.
[b] GacA has been previously identified and characterized (84).

TABLE 5. Putative wHTH RRs[a]

ORF	Length (amino acids)	Identity to CheY (%)	Conserved residue						Known or possible identity	% Identity/ similarity	Possible SK partner(s)
			D12	D13	D57	T87	Y106	K109			
VF0114	239	26.4	X	X	X	X	X	X	OmpR	83/94	VF0115 (EnvZ)
VF0526	226	21.7		X	X	X	X	X			VF0524, VF0525
VF1396	218	24.8	X	X	X		X	X			VF1397
VF1570	232	24.8		X	X	X	X	X	TorR	56/72	VF1619 (TorS)
VF1988	230	27.1		X	X	X	X	X	PhoB	80/91	VF1987 (PhoR)
VF2120	239	25.6		X	X		X	X	ArcA[b]		VF2122 (ArcB)
VF2343	227	26.4	X	X	X	X	X	X	CpxR	60/78	VF2344 (CpxA)
VFA0179	226	24.0		X	X	X	X	X			VFA0178
VFA0181	221	26.4	X	X	X	X		X			VFA0182
VFA0211	228	30.2		X	X		X	X			VFA0212
VFA0266	221	26.4		X	X	X	X	X			VFA0265
VFA0436	225	31.0		X	X		X	X			VFA0435
VFA0561	218	22.5	X	X	X	X	X	X			VFA0560

[a] See Table 3 footnotes for explanations of column heads.
[b] ArcA has been previously characterized (8).

(a possibility for numerous RR genes disrupted in this study), we will refer to our mutants simply as RR mutants; future work such as complementation and genetic analyses of the operon structure will be necessary to confirm the specific roles of RRs identified in this survey. Potential mutants were isolated by selection for Ery^r and subsequently confirmed using Southern blot analysis (see Materials and Methods). Resistant colonies arose at a frequency generally proportional to internal fragment length (i.e., larger fragments typically resulted in more-numerous resistant exconjugants). Although rarely, Southern analysis indicated that some isolates appeared to contain two copies of the suicide vector integrated in the RR gene. These strains were not used for further study. We constructed mutations in 35 of the 39 targeted RR genes in this manner. The remaining four RR genes (VF0923, VF1830 [*cheB*], VFA0436, and VFA0608) were recalcitrant to disruption despite multiple attempts; further experiments are required to determine whether these represent essential genes.

Since this method of making mutations has been used to a limited extent in *V. fischeri* and certainly has not been utilized on such a large scale, some additional characterization of this technique was warranted. First, we probed the lower size limit of homologous DNA that could be recombined successfully into the *V. fischeri* chromosome. We generated derivatives of our *arcA* mutant construct that truncated the original 314-bp gene fragment to 101 bp and 85 bp and introduced them into *V. fischeri*. ERY-resistant colonies were rare but arose with greater frequency with prolonged periods of conjugation. Southern analysis verified appropriate chromosomal insertion of the smaller *arcA* constructs in KV1703 and KV1704. In addition, we found that these strains (as well as our original *arcA* mutant, KV1548) exhibited increased luminescence (Fig. 2A), consistent with the function of ArcA as a negative regulator of bioluminescence (8). These results demonstrated that an 85-bp fragment is sufficient for homologous recombination in *V. fischeri*; thus, this method can be readily utilized for constructing mutations in small genes.

Second, we evaluated the stability of the *V. fischeri* vector integration mutants, as homologous recombination can occur to remove the partial duplication in these strains. We tested stability by growing three representative mutants for at least 10 generations in the absence of selective pressure. The three mutants contained duplications arising from recombination of 85-, 306-, and 651-bp regions of DNA (in *arcA*, VF0526 [*phoP*] and VF1854 [*flrC*], respectively). In two experiments, 100% of >200 colonies of each mutant strain remained resistant to ERY, suggesting that this type of mutation in *V. fischeri* is relatively stable and that constant selective pressure is unnecessary. Thus, the majority of subsequent experiments were carried out in the absence of selective pressure.

TABLE 6. Putative σ^{54}-dependent RRs[a]

ORF	Length (amino acids)	Identity to CheY (%)	Conserved residue						Known or possible identity	% Identity/ similarity	Possible SK partner(s)
			D12	D13	D57	T87	Y106	K109			
VF0095	467	28.7	X	X	X	X	X	X	NtrC	69/80	VF0096 (NtrB)
VF0454	445	17.1			X				VpsR[b]	67/84	Unpaired
VF0937	476	26.4		X	X	X		X	LuxO		VF0708, VF1036[c]
VF1401	475	25.6	X	X	X	X		X			VF1400
VF1854	477	31.8		X	X	X	X	X	FlrC[b]	78/85	VF1855 (FlrB)
VFA1026	500	21.7		X	X	X		X	SypG[d]		VFA1025 (SypF)

[a] See Table 3 footnotes for explanations of column heads.
[b] Compared to *Vibrio cholerae* proteins VpsR (88) and FlrC (34).
[c] LuxO likely functions in a phosphorelay, receiving its phosphate from phosphotransferase protein LuxU (VF0938), which likely responds to two sensor kinases, LuxQ (VF0708) and AinR (VF1036), a LuxN homolog (42).
[d] SypG has been previously named (90).

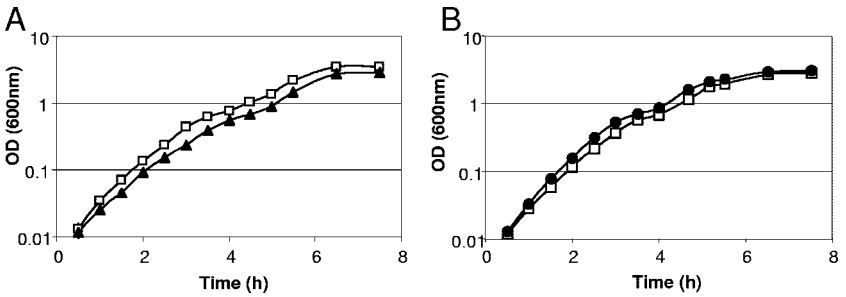

FIG. 1. Growth of RR mutants in culture. Growth of the control strain KV1421 (open squares) was measured in SWT medium over time and compared to growth of the *arcA* (A) (closed triangles) and VF1854 (*flrC*) (B) (closed circles) RR mutants. Experiments were performed in triplicate, and error bars are present but obscured by the icons representing each data point.

Impact of RR mutations on growth and luminescence. One of the hallmarks of *V. fischeri* biology is the ability to grow to high cell density and produce light. To address the involvement of two-component pathways in this process, we analyzed the ability of the 35 RR mutants to grow and luminesce. Because we have previously shown that the presence of the Ery^r marker decreases the luminescence and motility of *V. fischeri*, even in the absence of selection (58), we compared the growth and luminescence of the Ery^r RR mutants to those of KV1421, a derivative of the wild-type strain also marked with Ery^r. Upon plating of each mutant on solid medium composed of either the complex medium SWT or MM supplemented with a variety of carbon sources, we observed that only the *arcA* mutant generated consistently smaller colonies than did the control strain, KV1421. We further examined each of these strains in liquid SWT medium and found that most grew at a rate indistinguishable from that of the control. However, consistent with the observations of growth on solid medium, the *arcA* strain exhibited a significant, yet minor, deficit in growth, as measured by OD in SWT broth compared to that of the control (Fig. 1A). This result is consistent with previous observations (8). Conversely, a strain carrying a mutation in the putative homolog of the flagellar regulator *flrC* exhibited consistently higher OD measurements than did the control, indicating a possible growth advantage (Fig. 1B).

Bioluminescence of *V. fischeri* occurs at high cell density in response to the synthesis, release, and uptake of autoinducers (19–21). To date, the only RRs associated with luminescence are the negative regulators LuxO and ArcA (8, 52). To investigate whether other two-component regulators affect this pathway, we measured the bioluminescence of each mutant strain in SWTS medium relative to the control, KV1421. The growth characteristics of each RR mutant in SWTS generally paralleled those observed in SWT (data not shown). To account for any minor differences in growth or starting inoculum, we analyzed the specific luminescence of each strain relative to the OD. Representative experiments are depicted in Fig. 2.

Consistent with previous observations, mutations in *arcA* and *luxO* resulted in increased bioluminescence at high cell density (Fig. 2A) (42). Three different mutants defective for the RR ArcA displayed high levels of luminescence; all reached a level above the limit of detection at cell densities near or above an OD_{600} of 2. Additionally, the *luxO* mutant consistently achieved specific luminescence levels about fourfold above that of KV1421 (Fig. 2B). With few exceptions, most other RR mutant strains induced light production at rates similar to those of the wild type and achieved the same maximal levels (data not shown). For example, a mutant defective for the RR SypG, which exhibits substantial sequence similarity to the luminescence regulator LuxO, was indistinguishable from KV1421 (Fig. 2C). However, we did identify three RRs that appeared to enhance bioluminescence, as mutations that disrupted VFA0698 (*cheV*), VF1854 (*flrC*), or VF1148 (*yehT*) caused a small but reproducible decrease in bioluminescence levels compared to the control (Fig. 2D, E, and F, respectively). These data thus reveal additional minor players (either direct or indirect) in bioluminescence control.

Role of RRs in motility. In many bacteria, numerous environmental factors influence bacterial motility, making it another phenotype likely controlled by two-component regulation in *V. fischeri*. Therefore, we examined the motility of the 35 RR mutants by inoculating TB-SW soft agar plates each with an RR mutant and a control and measuring the diameter of the outer chemotaxis ring over time. Again, many RR mutants, such as the *sypG* mutant (Fig. 3A), exhibited no significant defect in motility compared to the control. However, we identified three classes of motility phenotype mutants: (i) those that appeared nonmotile, (ii) those with decreased migration, and (iii) those with increased migration relative to the control.

The first class of mutants, those that appeared nonmotile, included two strains (Fig. 3B). One was disrupted for VF1854, which encodes a putative homolog of *V. cholerae* FlrC, a protein necessary for flagellar gene expression (13, 60). The other contained a mutation in VF1833, the putative *cheY* gene. Because mutations in *cheY* are expected to disrupt chemotaxis rather than motility, we used a light microscope to examine the motility of these two strains and the control following growth in a liquid medium. We found that, whereas cells defective for the *flrC* homolog were nonmotile, those of the putative *cheY* mutant and the control were motile. Furthermore, our observations suggested that the *cheY* mutant cells exhibited far less frequent reversals than did the control (KV1421) cells, consistent with the smooth-swimming phenotype of typical *cheY* mutants. These data thus support the identification of VF1833 as *cheY* and of VF1854 as *flrC*.

The second class of mutants included five strains with decreases in migration (Fig. 3C). Two of these were strains defective for *arcA* and *luxO*. The *arcA* mutant (Fig. 3C, closed diamonds) exhibited a slight yet significant defect in migration rate. This is a phenotype not previously reported for this reg-

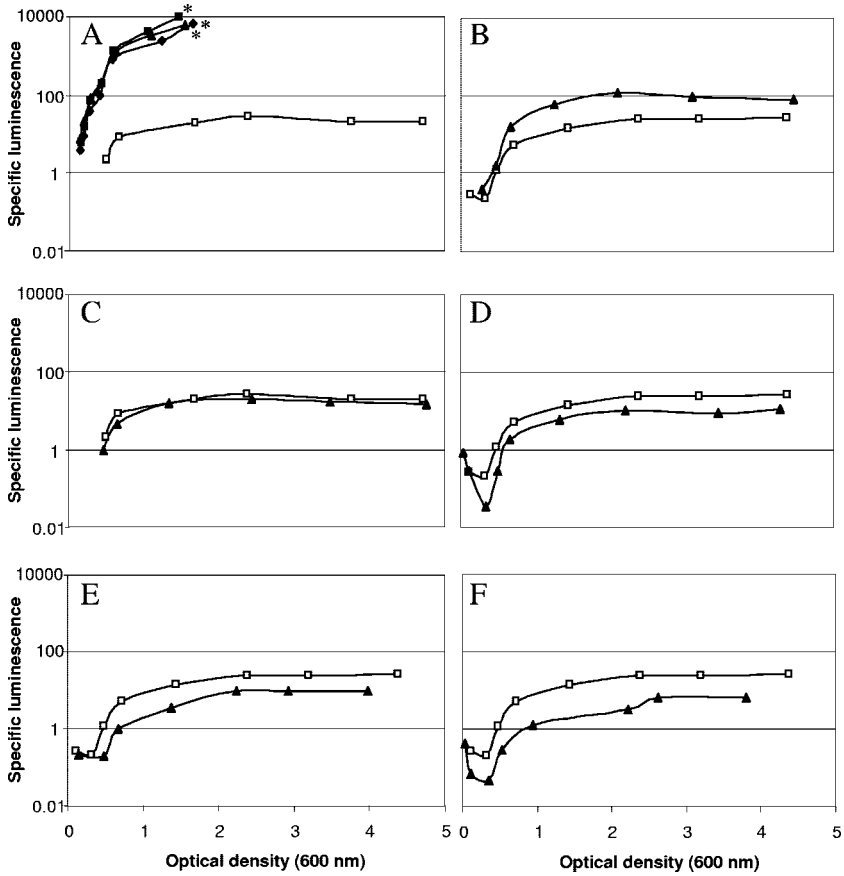

FIG. 2. Luminescence of RR mutants in culture. Specific luminescence (relative luminescence/OD_{600}) versus OD_{600} for KV1421 (open squares) was compared to *arcA* mutants (KV1548, KV1703, and KV1704; closed triangles, closed squares, and closed diamonds, respectively) (A), each of which reached levels of luminescence above the limit of detection (*), as well as to strains lacking the following RRs: *luxO* (B), *sypG* (C), VFA0698 (*cheV*) (D), VF1854 (*flrC*) (E), and VF1148 (*yehT*) (F). Shown are representative data from one of four independent experiments.

ulator; however, given the growth defect of this strain, verification of this result will require a growth-independent means of validation not part of this study. The *luxO* mutant (Fig. 3C, open triangles) exhibited the most severe motility defect of this class, with a greater-than-twofold decrease in the rate of motility on TB-SW soft agar plates relative to the control. This phenotype is consistent with that previously reported for a *luxO* mutant (41).

The second class of mutants also included those defective for VF0454 (*vpsR*) (Fig. 3C, closed squares), VF1909 (*narP*) (Fig. 3C, closed circles), and VF2343 (*cpxR*) (Fig. 3C, closed triangles). Disruption of VF2343 resulted in a slightly decreased rate of motility compared to the control. The two remaining strains were delayed in migration; each exhibited a strongly decreased rate of migration compared to that of the control until about 3 h postinoculation.

The third class of mutants contained a single strain, defective for VFA0698, which exhibited a small but reproducible increase in motility relative to the control (Fig. 3D). Intriguingly, VFA0698 is one of three RRs that exhibit sequence similarity to *cheV* (Table 3). In summary, these experiments supported the identification of LuxO as a motility regulator and revealed up to seven additional regulators that impact motility; additional experiments will be required to determine the mechanisms by which these RRs affect motility.

Multiple RRs play a role in colonization. Two-component regulators often mediate a bacterium's ability to sense and respond to host-specific signals during colonization. In fact, at least three such regulators, the RRs GacA and LuxO and the SK RscS, are known to affect the ability of *V. fischeri* to initiate colonization of the squid host (41, 79, 83, 84). To characterize our RR mutants with respect to symbiosis, we assayed the ability of the mutants to compete with the wild-type strain for colonization. In these experiments, we inoculated squid with approximately 1:1 mixtures of each RR mutant and a wild-type control. We evaluated successful colonization by measuring bioluminescence, which occurs when the bacteria have achieved a high cell density within the symbiotic light organ. We then isolated bacterial symbionts from colonized squid and determined the relative amounts of each of the two strains. To facilitate differentiation of the two strains, we marked one of the two strains using a stable plasmid that constitutively expresses *lacZ*, which does not affect competitive colonization (18), and plated the strains on medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Finally, we calculated the RCI by normalizing the

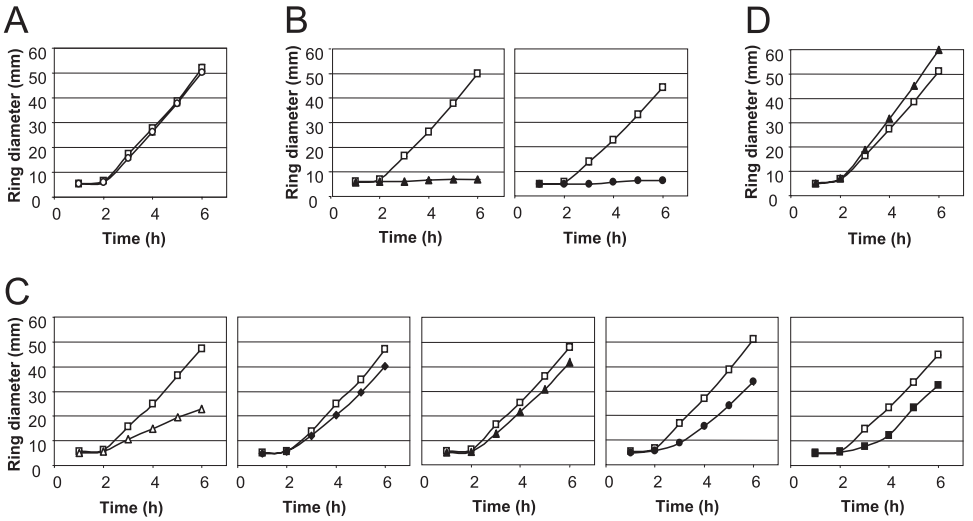

FIG. 3. Motility of RR mutants. Migration of KV1421 (open squares) was assayed over time in TB-SW soft agar plates by measuring the diameter of the outer motility ring and compared to that of strains lacking *sypG* (open circles) (A); *flrC* (closed triangles) and *cheY* (closed circles) (B); *luxO* (open triangles), *arcA* (closed diamonds), VF2343 (*cpxR*) (closed triangles), VF1909 (*narP*) (closed circles), and VF0454 (*vpsR*) (closed squares) (C); and VFA0698 (*cheV*) (closed triangles) (D). Experiments were conducted in triplicate, and error bars are present but obscured by the icons representing each data point.

ratio of output bacteria to the ratio of the input bacteria, which we report as a $\log_{10}$-transformed value (log RCI) for purposes of comparison (see Materials and Methods). A log RCI value below zero indicates a competitive disadvantage for an RR mutant; we report here only those mutants with a mean log RCI significantly less than zero as evaluated by Student's *t* test.

Of the 35 mutants that we tested, 23 exhibited no competitive defect in colonization. For example, squid inoculated with a strain defective for VF1396 consistently exhibited a log RCI of zero or greater (Fig. 4A). In contrast, 12 of the mutants tested exhibited a significant defect in competitive colonization in at least two independent experiments (Fig. 4B to D and data not shown). Subsequently, these 12 strains were also tested for competitive growth in culture. Most of the RR mutants that were competitively defective for colonization did not exhibit growth disadvantages in coculture (data not shown). Those that did exhibit statistically significant coculture differences are reported below.

Several mutants with defects in motility exhibited competitive defects (Fig. 4B). A mutation in *luxO*, a gene previously shown to be necessary for symbiotic initiation (41), resulted in a substantial competitive disadvantage (Fig. 4B, average log RCI of <-1.48). In addition, the *flrC* mutant was among the most severely compromised strains, as the inoculated squid yielded levels of mutant bacteria below our limit of detection (14 CFU/squid). It is likely that the lack of motility in this strain accounts for its colonization defect, as has been shown for other flagellar mutants (25, 49, 50). In coculture, the *flrC* mutant exhibited a competitive growth advantage in rich medium (data not shown), a phenotype that is unlikely to account for its colonization defect. A mutant defective for *cheY* was also severely defective in competition during colonization; a potential role for chemotaxis in symbiotic association will be discussed below. Although the *cheY* mutant exhibited a slight competitive growth disadvantage (average log RCI of -0.2286, $P = 0.0357$), this phenotype is unlikely to account for the severe colonization defect that we observed. Finally, three other RR mutants that displayed less-severe changes in motility also exhibited competitive defects: VF0454 (*vpsR*), VF1909 (*narP*), and VFA0698 (*cheV*). Notably, the *cheV*-like VFA0698 mutant (average log RCI of -0.8) exhibited increased motility, as well as decreased luminescence.

We also found five mutants that exhibited no defects in growth, luminescence, or motility and yet failed to compete effectively for symbiotic colonization (Fig. 4C). They were disrupted for the following RR genes: VF0095 (*ntrC*), VF1689 (*expM*), VF1988 (*phoB*), VFA0179, and VFA0181. Of these, only the *ntrC* mutant exhibited a slight competitive growth disadvantage in coculture experiments (average log RCI of -0.553, $P = 0.0018$). Further studies will be required to identify the pathways governed by each of these RRs and to determine whether they represent previously uncharacterized colonization determinants.

Finally, we also found that a strain that lacked the RR SypG was severely defective in competition. SypG has been shown to regulate transcription of a cluster of genes, *syp*, which are required for colonization (90); however, no mutations in *sypG* have previously been characterized. As shown in Fig. 4D, of nine animals inoculated with labeled ES114 and the *sypG* mutant, all were exclusively colonized with ES114.

Because SypG was the only RR that was completely excluded from the light organ and exhibited no motility or luminescence defect, we characterized *sypG* further by constructing an in-frame mutation to eliminate potential polar effects on the downstream gene *sypH*. We tested the Δ*sypG* mutant in single animal inoculations and found that under these conditions, the Δ*sypG* strain was severely defective in initiating symbiosis compared to the wild-type strain (a representative experiment is depicted in Fig. 5; $P = 0.01$). In fact, 5 of the 10 animals inoculated with the Δ*sypG* strain yielded no bacteria upon

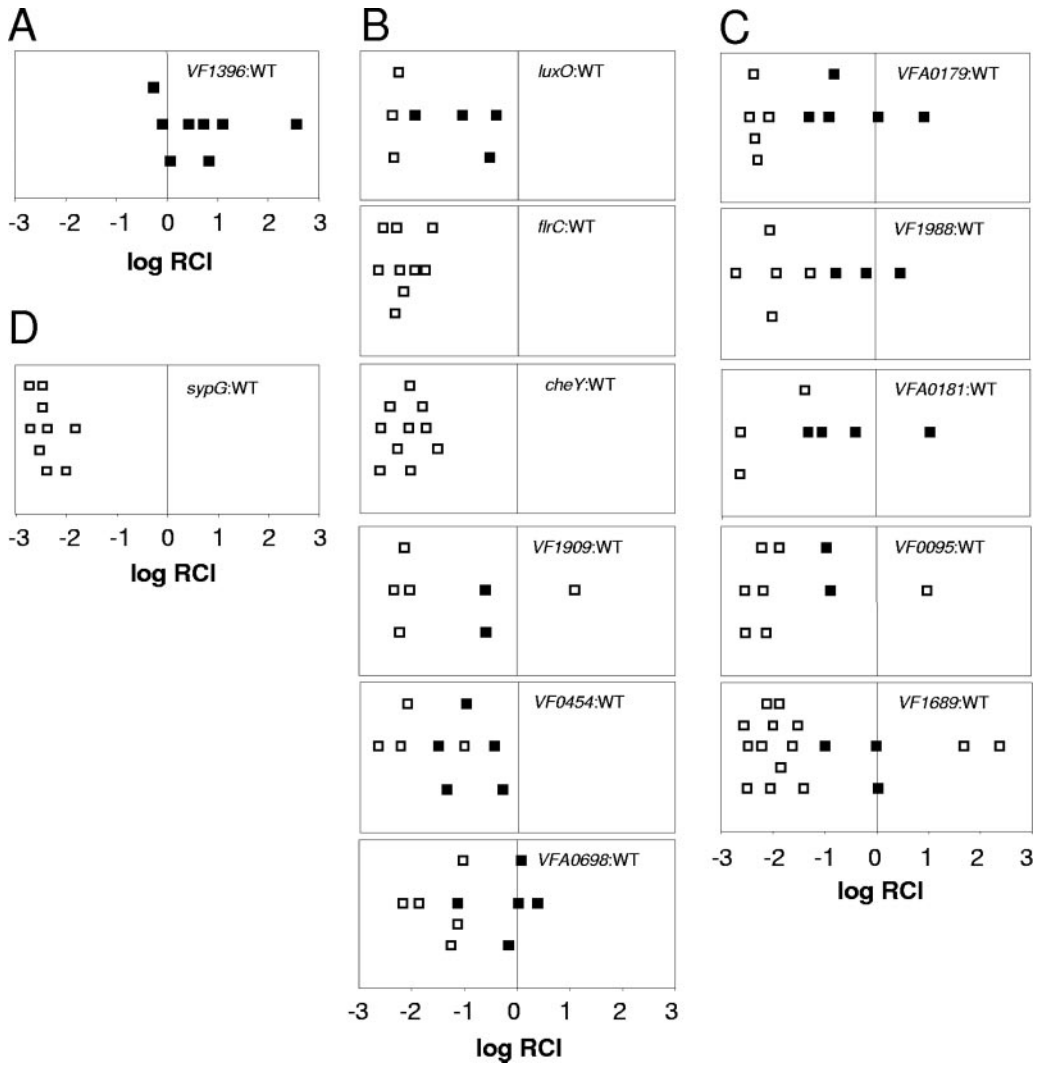

FIG. 4. Competitive colonization of RR mutants. Juvenile squid were coinoculated with RR mutant and marked wild-type (ES114) *V. fischeri* strains for approximately 18 h. The animals were then homogenized, and colonization was measured by the subsequent determination of the mutant-to-wild-type (WT) ratio of bacterial cells present within each squid (reported as log RCI; see Materials and Methods). A log RCI of <0 indicates that wild-type bacteria outnumber the mutant bacteria, indicating a competitive defect, whereas a log RCI of >0 indicates that mutant bacteria outnumber wild-type cells. Open squares represent animals in which either the mutant (log RCI, <0) or wild-type (log RCI, >0) cells were below the limit of detection (14 CFU/squid, in these experiments). Positioning along the *y* axis is arbitrary. (A) VF1396 mutant; (B) mutants which affect known symbiosis determinants (motility and/or luminescence); (C) strains which do not affect motility or luminescence; (D) a strain lacking *sypG*, which regulates the known symbiosis determinants within the *syp* cluster of genes.

homogenization (below the limit of detection, 14 CFU/squid). These data thus supported the prediction that SypG would be an important symbiotic regulator (90) and also revealed the utility of performing competition experiments to identify genes that might be necessary for symbiotic colonization even in the absence of competition.

DISCUSSION

To date, global genetic analyses and characterization of two-component signal transduction systems have been conducted in relatively few bacteria, largely in model organisms such as *Escherichia coli*, *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* (27, 35, 36, 53, 77, 87). This work, conducted in the marine bacterium *V. fischeri*, adds to this relatively small data set. Through a bioinformatics approach, we identified 40 putative two-component RRs encoded by *V. fischeri* based, in part, on the presence of a canonical REC domain. Using a recently developed suicide plasmid and a vector integration approach, we subsequently mutated 35 of these regulators. We then examined several phenotypes in culture as well as the impact of these mutations on symbiotic colonization through competition analyses. This work demonstrated the feasibility of such a large-scale mutagenesis in *V. fischeri* and resulted in the identification of novel regulators of motility, bioluminescence, and colonization.

To identify RRs, we relied on bioinformatics approaches, searching the ERGO Light database (http://www.ergo-light.com/ERGO/) and then analyzing the candidates using rps-

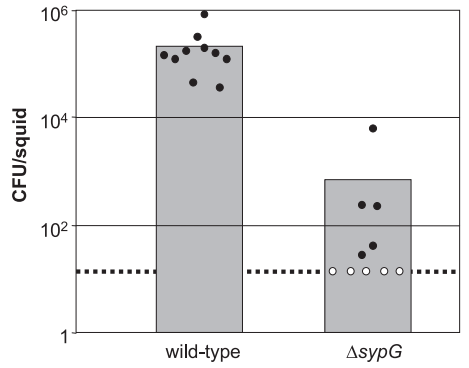

FIG. 5. Individual colonization of wild-type and Δ*sypG* strains. Individual animals (open or closed circles) were inoculated with wild-type (ES114; 1,390 cells/ml) or Δ*sypG* mutant (2,610 cells/ml) strains of *V. fischeri* for approximately 18 h and subsequently homogenized. Bacterial numbers were assessed by plating and are reported as CFU per squid. Open circles represent homogenates containing fewer than 14 CFU per squid (our limit of detection; dashed line). Gray boxes represent the average CFU for each strain of bacteria.

BLAST (44). We subsequently compared our results with data recently made available through the Center for Biological Sequence Analysis (CBS; http://www.cbs.dtu.dk/), which lists 47 putative RRs. The RRs that we identified through our analysis matched those listed by CBS, with the following exceptions. First, CBS listed 13 ORFs that we eliminated from our study, because further analysis indicated that those 13 ORFs contain both REC domains and sensory domains (i.e., the H box), and thus, they likely function as hybrid sensor kinase proteins rather than RRs. In addition, we identified five genes not listed by CBS. Four of these are CheY-like regulators that lack a DNA-binding domain: VF0923, VF1054, VFA0216, and VFA1017. Each of these contains a REC domain and most of the highly conserved residues therein (Table 3). We also included in our analysis VF0454 (*vpsR*), although it is unclear whether this regulator will indeed function as a RR. Alignments of this protein with LuxO (VF0937) revealed an overall conservation within the REC domain of the latter protein, supporting this assignment. However, like its *V. cholerae* homolog (88), VF0454 (*vpsR*) lacks several of the highly conserved residues in the REC domain (Table 6). Because our identifications were based on bioinformatics rather than biochemical analyses, further studies will be necessary to verify the function of these putative RRs as phosphate-accepting two-component proteins.

Our vector integration approach to interrupt the RR genes successfully yielded disruptions in 35 of the 39 RRs that we targeted for mutation with relative ease. As controls, we disrupted two previously characterized RRs, *luxO* and *arcA*. These mutations each resulted in phenotypes consistent with previous reports (8, 40–42). We can conclude, therefore, that our mutagenesis approach is capable of generating null mutations. Because many of the predicted RRs disrupted in this work appear to be encoded within operons (data not shown), the specific roles of these regulators must be addressed by complementation experiments in future work. Regardless of this caveat, this study reveals novel loci affecting downstream phenotypes such as colonization. It remains unclear why we were unable to obtain mutations in the remaining four genes. These may represent essential genes; however, this seems unlikely to be the case for VF1830 (*cheB*), which is predicted to play a role in chemotaxis.

Finally, we assessed the roles of the 35 RRs in two primary physiological traits in culture, bioluminescence and motility, as well as in the ability to compete with a wild-type strain for symbiotic colonization. We identified eight RR mutants with motility phenotypes, five with bioluminescence phenotypes, and 12 with symbiosis phenotypes. Of the symbiosis-defective strains, six exhibited alterations in either motility or bioluminescence or both, while six were wild type for these phenotypes. RRs required for symbiosis were found on each of the two chromosomes, with proportionally more of them mapping to the larger chromosome. They were also found in each of the four RR classes (regulators with no predicted DNA-binding sequences, with HTH or wHTH, or with σ^{54} activation domains). Intriguingly, of the six RRs that comprise the subgroup of σ^{54}-dependent RRs, five played roles in symbiosis, indicating the importance of this regulatory subclass in symbiotic colonization.

Of the six mutants defective for both symbiosis and other traits, the nonmotile *flrC* (VF1854) mutant is perhaps easiest to interpret: motility is essential for the initiation of symbiotic colonization (25, 49, 50), as demonstrated by the lack of *flrC* mutants found in the light organ in our experiments (Fig. 4B). A *cheY* mutant was similarly excluded from the light organ. This mutant also failed to migrate in soft agar plates; however, consistent with the predicted role of CheY as a chemotaxis regulator, the *cheY* mutant was actually motile but exhibited predominantly smooth swimming in liquid medium. These experiments indicate that chemotaxis may be important for symbiotic colonization. In support of this, we have similarly found that a mutant defective for *cheR*, another chemotaxis regulator whose loss results in smooth swimming, was also defective for colonization (14a). However, since both *cheY* and *cheR* mutants fail to migrate through soft agar, it remains formally possible that these strains fail to colonize not due to a lack of chemotaxis but due to an inability to penetrate the squid-secreted mucus layer. It will be necessary to construct specific chemoreceptor mutants or identify a squid-secreted chemoattractant to fully address this question.

Of the five additional mutants that exhibited decreases in motility, three also displayed symbiosis phenotypes (VF0454 [*vpsR*], VF1909 [*narP*], and *luxO*). Thus, it is tempting to speculate that reduced motility reduces colonization proficiency. This appears not to be true, however, in the case of the VF2343 and *arcA* mutants, which exhibited decreased motility but remained proficient for colonization. In addition, an interpretation for the *luxO* mutant, which was among the most severely compromised for motility, is complicated by the role which this regulator plays in the control of bioluminescence and other genes (40–42, 52).

Motility and colonization are also linked in the case of the mutant disrupted for VFA0698 (*cheV*). In this case, however, the mutant displayed an increase in motility. It also exhibited a decrease in bioluminescence. VFA0698 is one of three RRs predicted to encode a CheV-like protein, with both REC and CheW domains. In *Bacillus subtilis*, CheV functions to couple methyl-accepting chemotaxis proteins to the SK CheA, allow-

ing chemotactic signaling to occur (33, 62). Further experiments are required to determine whether any of the three *V. fischeri* CheV-like proteins performs such a function. The relative contribution to the symbiotic defect, if any, of the bioluminescence and motility phenotypes of the VFA0698 mutant remains unclear.

Perhaps most exciting are the six RRs whose loss caused a symbiosis defect but no alterations in bioluminescence or motility: VF0095 (*ntrC*), VF1689 (*expM*), VF1988 (*phoB*), VFA0179, VFA1081, and *sypG*. Of these, the most severe was the *sypG* mutant, which was excluded from the light organ. Because the insertional mutation in this strain could cause polar defects on a downstream gene, we further investigated the role of *sypG* by constructing an in-frame deletion and evaluating its ability to colonize juvenile squid in single-inoculation experiments. The results confirmed that this regulator plays an important role in symbiotic initiation (Fig. 5). The *sypG* gene resides within a large cluster of genes that are necessary for symbiotic colonization and function in polysaccharide biosynthesis (90). Our recent work demonstrates that *sypG* overexpression induces transcription of the *syp* genes, making it likely that this regulatory role accounts for the symbiotic requirement for SypG (90). Whether SypG controls additional colonization factors remains to be determined.

Of the remaining five RRs important for colonization, roles for VF0095 (*ntrC*), VF1689 (*expM*), and VF1988 (*phoB*) can be hypothesized based on bioinformatics. For example, VF1988, a putative PhoB homolog, could play a role in controlling genes based on phosphate availability. Similarly, the possible NtrC homolog VF0095 may respond to changing nitrogen levels. VF1689 may be a homolog of ExpM in *Erwinia carotovora*, which does not bind DNA but rather impacts the stability of the stationary-phase sigma factor σ^S (2). The remaining regulators (VFA0179 and VFA0181) contain predicted DNA-binding domains, which will likely facilitate investigations into their functions. Intriguingly, these two regulators are encoded near one another on the chromosome. These genes might thus represent a novel symbiosis locus.

Many of the RR mutants exhibited no defects or relatively minor defects in our competitive colonization experiments; however, this does not rule out the possibility that other RRs function in symbiosis. Whereas the competitive colonization experiments described herein were terminated 24 h after inoculation, other similar assays described in the literature are often extended to 48 h to account for the ability of a given bacterial strain to persist within the light organ after initial colonization. Thus, those RRs that are essential for persistence may not be defective (or may be only slightly defective) during the initial 24 h of symbiosis. Indeed, although we found no significant competitive defect for an *arcA* mutant at 24 h, recent work indicates that an *arcA* mutant exhibits a defect within 48 h (8). Additionally, it is possible that mixed inoculation with wild-type strains may complement some RR mutant phenotypes required for colonization. For example, an RR mutant unable to produce an unknown extracellular symbiosis determinant may be able to obtain that determinant from its wild-type neighbors during inoculation, thus masking a colonization phenotype. Thus, future experiments may identify additional RRs that function in symbiosis.

In summary, our results highlight the utility of a global approach for investigating gene function in *V. fischeri*. Together these experiments have provided a wealth of information regarding the roles of *V. fischeri* RRs in a variety of phenotypes, including symbiotic colonization. These results will serve as a starting point for understanding the connections between these global regulators and the physiological traits which they impact, particularly as microarray technology is applied to the mutant strains generated here.

ACKNOWLEDGMENTS

We thank Alan Wolfe, Jonathan Visick, and members of the Visick lab for critical reading of the manuscript.

This work was supported by NIH grants RR12294, awarded to E.G.R., and GM59690, awarded to K.L.V.

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